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# Stimulatory Effect of 660 nm Low Level Laser Energy on Hypertrophic Scar-derived Fibroblasts: Possible Mechanisms for Increase in Cell Counts

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***Background and Objective:*** **Varying effects of red light wavelengths on in vitro cells have been reported. Low level lasers (LLL) are employed to assist wound healing especially for indolent ulcers. On healing, burn wounds may become hypertrophic, resulting in excessive wound contraction, poor cosmesis, and functional impairment. This study enquired whether 660 nm LLL affected hypertrophic scar-derived fibroblasts.**
***Study Design/Materials and Methods:*** **The experiments investigated the effect of a 660 nm, 17 mW laser diode at dosages of 2.4 J/cm$^2$ and 4 J/cm$^2$ on cell counts of two human fibroblast cell lines, derived from hypertrophic scar tissue (HSF) and normal dermal (NDF) tissue explants, respectively. The protocol avoided transfer of postirradiated cells. Estimation of fibroblasts utilized the methylene blue bioassay.**
***Results/Conclusion:*** **The post-660 nm-irradiated HSFs exhibited very significantly higher cell counts than controls $P < 0.01$ on days 1–4 (Mann-Whitney *U*-test), and $P < 0.01$ on days 1–3 for similarly irradiated NDFs. Lasers Surg. Med. 22:294–301, 1998.**
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**Key words: in vitro fibroblasts; LLLT; red light**

## INTRODUCTION

The biomodulatory effect of low level lasers (LLL), i.e., power emitted <500 mW, on cell functions, e.g., on change in cell numbers and the rate of collagen synthesis of normal human fibroblasts in cultures, have been reported previously [1–8]. The majority of these studies have demonstrated stimulatory effects of LLL on cell functions. Of the studies that assessed cell numbers following irradiation by mainly monochromatic red light, usually from a HeNe laser, several showed increased cell numbers [1,4,5,7,9], including fibroblasts cell lines [1,5,7,9], whereas fewer studies [2,6,10] found that HeNe had no effect on cell numbers.

In some instances, wounds, especially burn wounds, heal in a manner resulting in hypertrophic scars, indicative of altered behaviour of wound fibroblasts, which play an essential role in fibroplasia during the wound healing process [11]. Increased cell numbers, greater collagen deposition rate, or decrease in collagenase activities is likely to contribute to this abnormal repair process, characterized by exuberant dermal fibrosis and scarring. Fibroblast cell counts or numbers at specific intervals indicate their mitotic levels. Al-

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though an increased rate of fibroblast mitosis may not be related to greater collagen production or a decrease in collagenase activities, nevertheless, due to their greater numbers, their potential for enhanced collagen production collectively may be reasonably expected to increase.

Red light wavelengths have been used clinically on wounds to promote healing, but it is not known from previous work whether they also increase fibroplasia excessively [12,13], which can be counterproductive, especially in wounds that are susceptible to hypertrophic healing. Thus energy densities within the "therapeutic window" of up to 4 J/cm$^2$ [14,15] have been frequently employed clinically in the treatment of wounds and ulcers [12,13]. To gain better insight into the effect of red light on burn wound fibroblasts, this study tested for differences, if any, in cell counts following 660 nm LLL of 2.4 J/cm$^2$ and 4 J/cm$^2$ from a 17 mW laser diode on postburn hypertrophic scar-derived fibroblasts (HSFs).

The energy density is a measure of the intensity of energy being deposited in a cell-suspension (J/cm$^2$). It is the product of the power density (W/cm$^2$) and the duration of irradiation in seconds. The energy densities employed in most of the cell studies have revealed stimulatory effects that were 4 J/cm$^2$ or less, and often, below 2 J/cm$^2$.

As in any in vitro study, there are some limitations because of the difficulties in extrapolating results to the in vivo state. However, the methodology can be highly controlled compared to clinical trials, in which ethical and other issues may not be readily addressed, or to animal studies, due to the lack of a readily available animal model able to exhibit human-like hypertrophic scars.

## MATERIALS AND METHODS

Small biopsies of hypertrophic scar tissue were obtained under aseptic operating conditions from a 20-year-old Chinese female patient who was undergoing corrective surgery on the neck-shoulder region. The excised tissue biopsies used were from unwanted tissue that would have been discarded after the operation. The explant technique protocol for establishing the primary culture of the HSF cell-line was a slight modification of that described by Freshney [16]. The tissue biopsies were removed from the primary wash of Dulbecco's modified Eagle's medium (DMEM) (Gibco) that had penicillin and streptomycin incorporated into it and were thoroughly rinsed with phosphate-buffered saline (PBS) (Oxoid). In a sterile petri dish containing 2 ml primary culture medium, the tissues were cut into fragments of <1 mm in any dimension. They were placed on the base of a 50 ml culture flask (Nunclon) and incubated with the flask inverted for 24 hours at 37°C in a humidified atmosphere containing 5% $CO_2$. At the end of this period, another 2 ml of culture medium was added to the flask and then incubated the right way up. The primary culture medium was DMEM (Gibco), supplemented with 10% foetal bovine serum, L-glutamine, penicillin at 100 units/ml, and streptomycin at 100 μg/ml (all from Gibco). With the first sign of cellular outgrowths from the explants, the primary culture medium was replaced and then changed after every 3 days.

After about 2 weeks when near confluency was achieved, short trypsinisation with 0.05% trypsin in EDTA (Gibco) was carried out. This selectively detached the fibroblasts from the other cell types that adhered more to the plastic substrate. The cell suspension was centrifuged, the supernatant removed, and the cells resuspended in fresh culture medium and subcultured in a fresh culture flask. These first passage cells were again subjected to short trypsinization of 6 minutes and resuspended in fresh culture medium. Subculturing was continued similarly, but longer trypsinization duration of 10 minutes was used subsequently. When the cells were at Passage 4, they were harvested for cryopreservation in liquid nitrogen. For the experiments, cells were retrieved from preservation, thawed out, and cultured as described above for two more passages, so that only Passage 6 HSFs were utilized. Apart from microscopic, morphological observations, the fibroblasts "homogeneity" was tested immunohistochemically. They were negative with: anticytokeratins to exclude keratinocytes; anti-von Willebrand (Factor VIII) in excluding endothelial cells; antidesmin in ensuring they were not smooth muscle cells, and they tested positively for monoclonal mouse antiswine vimentin antibody to demonstrate the cells are of mesodermal origin (all the antibodies and test-kit were from Dako Products, Japan).

For comparison with the effect of 660 nm LLL on the HSF cell-line, a normal dermal fibroblast cell line (NDF) was subjected to the same protocol. The NDF had been identically subcultured from normal dermal tissue biopsies taken from surgically discarded shoulder region material of another Chinese female adult whose scars

had not shown any evidence of hypertrophy. This method was chosen in preference to punch biopsies from normal dermal fibrous scars for ethical reasons.

The HSF proliferation bioassay involved inoculating 100 μl volumes of cell suspension into specific microculture wells of alternate columns (Linbro, Flow Laboratories). Thus each inoculated well contained ~1,000 cells in primary culture medium. Of the cell-containing wells, half were irradiated by the 660 nm laser diode (3 ML, Omega Universal Technologies) at an energy density of 2.4 J/cm$^2$, whereas the other half were sham-irradiated i.e., the laser diode was placed in position but not switched on (no light being emitted from it). The optical power output at emission from the laser diode was tested with an optical power meter (OLS, OPM93, Omega Universal Technologies) just before irradiation of the cell-suspensions. The distance from the tip of the 660 nm diode to the surface of the culture medium was 0.6 cm and the diameter of each microwell was 0.65 cm. The parameters of the LLL source were: (1) source: Noncoherent Omega superluminous diode; (2) average power output: 17 mW; (3) spot size at 0.6 cm distance: 0.217 cm$^2$; (4) source spectral bandwidth at 95% intensity: 5 nm; (5) total angle of divergence: 12°; (6) pulsing frequency: 5,000 Hz; (7) pulse duration: 160 μs; (8) power density at 0.6 cm distance: 0.078 W/cm$^2$; and (9) duration of exposure: 31 s for energy density of 2.4 J/cm$^2$ and 52 s for 4 J/cm$^2$.

After irradiation, the empty wells were filled with culture medium. All the marked and sealed culture plates were placed side by side on the same shelf of the incubator, set at 37°C with humidified atmosphere that contained 5% $CO_2$. The first plate was removed from the incubator and "stopped" 18 hours after irradiation. An additional plate was stopped each subsequent 24 hours.

Five microplates were prepared thus so that one plate could be stopped and processed on each of the 5 days after irradiation. All plates were bioassayed using a modified methylene blue method as described elsewhere [17] and optically read at 650 nm using a spectrophotometer (Anthos Labtec Instruments).

The experiment was then repeated using exactly the same protocol as described above and the same energy density. The entire experimental procedure was replicated with the energy density increased to 4 J/cm$^2$. Passage 6 NDFs were also subjected to an identical protocol as described for the HSF cell-line.

The diode laser equipment (3ML, Omega Universal Technologies) was new and used solely for the study. The equipment was thoroughly checked by the manufacturer's agent at regular intervals. The response power meter (OLS, OPM 93) was also serviced regularly and counter-checked for accuracy.

In order to convert absorbencies from spectrophotometric readings of the cell-containing wells into cell numbers in each experiment, the following procedure was carried out. Known numbers of serially diluted cells were seeded into specific wells of a microculture plate (Linbro) and incubated in a humidified atmosphere with 5% $CO_2$ at 37°C. The microculture plates were removed from the incubator after 4 hours and placed in a vertical laminar airflow tissue culture hood (Class II, Type A/B3, Nuaire). After carefully removing the culture medium with a minipipette, the monolayer of cells was rinsed with phosphate-buffered saline and fixed with 100% methanol. The culture plate was then sealed and stored so that it could be processed for the methylene blue bioassay contemporaneously with all the other plates used in the experiment. The assay involved staining the cells with the dye for 30 minutes, gentle rinsing thrice with 0.01 M borate buffer and elution of the dye with acid alcohol (1:1 ratio). As the specific cell numbers and their absorbency values were highly correlated, thus by linear regression, the absorbencies of the cell-containing wells were used to estimate cell numbers.

## RESULTS

The experimental findings revealed that 660 nm LLL had a stimulatory effect on the cell counts of both HSF and NDF cell-lines derived from biopsies of donors who were matched by race, gender, anatomical region, and age. Figures 1 and 2 illustrate the changes in cell numbers as compared to their respective controls, of the HSF and NDF cell-lines following irradiation by 660 nm wavelength, 17 mW power output, of energy density 2.4 J/cm$^2$, and 4 J/cm$^2$ respectively. In the figures, Experiments A (2.4 J/cm$^2$) and C (4 J/cm$^2$) denote the HSFs, whereas Experiments B (2.4 J/cm$^2$) and D (4 J/cm$^2$) were NDFs.

On days 1–4, following irradiation at 2.4 J/cm$^2$ and through the Mann-Whitney *U*-test (M-W *U*-test), the HSF cell counts were very significantly higher than their controls ($P < 0.01$), with

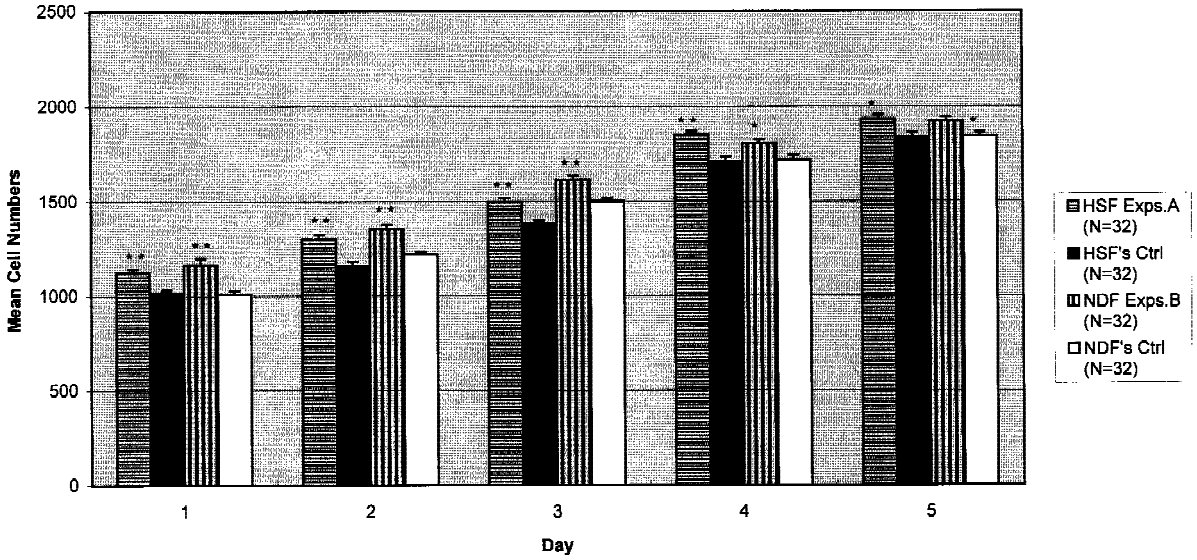



Fig. 1. **highly significant as compared to control ($P < 0.01$, M-W $U$-test). *significantly higher than control ($P < 0.05$, M-W $U$-test). Percentage increase in cell numbers of HSFs, postirradiation at 2.4 J/cm$^2$ as compared to respective control: day 1: 10.7%; day 2: 12.3%; day 3: 8.4%; day 4: 8.2%; day 5: 5.3%. Percentage increase in cell numbers of NDF as compared to control: day 1: 12.8%; day 2: 11.3%; day 3: 7.7%; day 4: 5.1%; day 5: 4.3%.

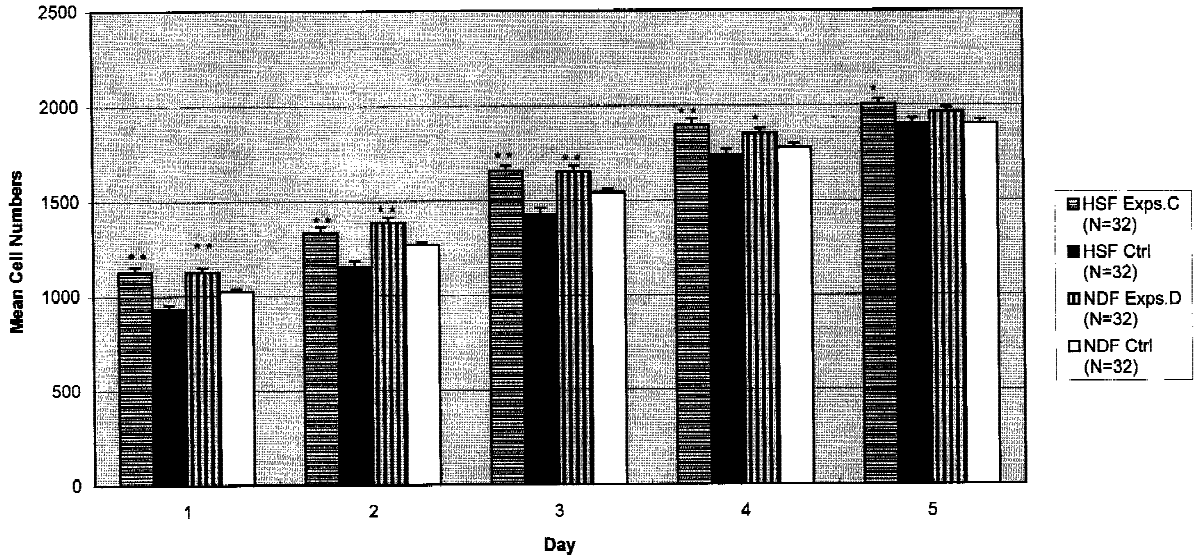



Fig. 2. **highly significant as compared to control ($P < 0.01$, M-W $U$-test). *significantly higher than control ($P < 0.05$, M-W $U$-test). Percentage increase in cell counts of HSFs, postirradiation at 4 J/cm$^2$ as compared to the respective control: day 1: 20.8%; day 2: 15.4%; day 3: 16.1%; day 4: 8.9%; day 5: 5.5%. Percentage increase in cell counts of NDFs, as compared to control: day 1: 10.2%; day 2: 9.1%; day 3: 7.1%; day 4: 4.3%; day 5: 3.4%.

the differences between them ranging from 8.2% to 12.3%. On day 5, the HSF cell count was still significantly higher than control ($P < 0.05$). The cell counts of similarly irradiated NDFs were very significantly higher than their controls on days 1–3 ($P < 0.01$) with their differences ranging from 7.7% to 12.8%, whereas on days 4 and 5, they were significantly higher than controls ($P < 0.05$); see Figure 1.

HSF cell numbers irradiated at 4 J/cm$^2$ were very significantly higher than controls ($P < 0.01$) on days 1–4 (differences of 8.9–20.8%) and still significantly higher ($P < 0.05$) on day 5. The 4 J/cm$^2$ irradiated NDFs were very significantly

higher than controls ($P < 0.01$) on days 1–3 (differences ranged from 7.1–10.2%) and significantly higher on day 4 ($P < 0.05$) only (Fig. 2).

## DISCUSSION

The results of this study and the possible biomechanisms involved are discussed in the context of other experimental findings of increased cell counts following red light irradiation and especially that of 660 nm. These suggest that photoenergy of 660 nm wavelength at the given parameters possibly induced the fibroblasts to secrete growth factors that probably acted in an autocrine manner to increase their rate of mitosis and/or reduce cell death. The stimulatory effect appeared slightly greater on the HSFs than on the NDFs.

Fibroblast, an important reparative cell together with other tissue repair cells and their cytokines, implement the healing process as demonstrated in an in vivo model [18]. Depending on the interval between irradiation and the biomodulation incurred, the growth factors could have served as competence factors for resting cells and as progression factors, promoting DNA synthesis in pre-mitotic, G1-staged cells. As the enhanced HSF and NDF cell numbers were very significant on postirradiation days 1–4 and days 1–3, respectively, may indicate that the stimulatory photoeffect had weakened over time especially with the NDFs.

### Stimulatory Effect of 660 nm LLL on Cells

It is known that significant levels of growth factors are present whenever cells are proliferating [19] and in support of this, is a study that demonstrated that 660 nm LLL at 2.16 J/cm$^2$ on 3T3 fibroblasts enhanced the cells' release of basic fibroblast growth factor (bFGF) [9]. The investigators in the same study [9] had transferred the supernatant from the irradiated fibroblasts on to bovine endothelial cells in vitro, which resulted in increased cell proliferation, hence questioning the belief that cultured cells lack the secretory signal peptides [20].

The FGFs, comprising at least nine homologous polypeptides and identified in several cells including fibroblasts, promote chemotaxis, proliferation and differentiation of wound healing cells [21]. They can be considered natural mediators of the tissue repair process since they stimulate the growth of fibroblasts, endothelial cells, Schwann cells, and chondrocytes [19]. FGF-1 and FGF-2 are considered essential in modulating dermal repair activities as they also enhance the chemotactic and mitogenic activities of vascular endothelial cells in vitro and are angiogenic in vivo [20]. However, the excessive production of FGFs may be associated with neoplasia [22]. If so, this has implications for the effect of low level laser therapy (LLLT) on human wounds, especially for those with a predisposition to hypertrophy. This is especially relevant considering growth factors are potent regulators of cell phenotype [23] and that fibroblasts were transformed into myofibroblasts by HeNe LLL [3]. It is not known if the parameters used by the authors promoted myofibroblast formation; this could be investigated by testing the cells for the presence of alpha-isoform of smooth muscle actin, present in myofibroblasts [3].

Further substantiation that LLL can induce cells to secrete growth factors is provided in another study [24], which demonstrated that polymorphonucleur leucocytes were stimulated to produce growth factors following 660 nm irradiation from a 15 mW GaAlAs diode pulsed at 5 KHz. Apart from the secreted FGFs effect on cell proliferation, other growth factors and biomechanisms are possibly present since in fresh wounds, other endogenous growth factors, notably PDGF and TGF-β [25,26], have been observed.

The observation made in this study agrees with others [27,28]. Bolton et al. [28] also utilized 660 nm LLL and found that it mediated the release of factors from macrophage-like, U-937 cells since their supernatant stimulated fibroblast proliferation significantly more than controls in a dose-response manner. As FGF-1, FGF-2, and other growth factors are synthesized by several wound healing cell-types including monocytes and macrophages [20], there are implications on the effect of 660 nm wavelength on wound healing. For example, after 660 nm-irradiation, significantly higher mast cell counts and their degranulation were demonstrated in partial thickness wounds of male Wistar rats [29]. The biologically active substances released by mast cells stimulate tissue repair, including its strong protective interaction between heparin-like molecules and FGF-1 and FGF-2 [30]. Thus the higher fibroblast counts observed in this study was induced probably by a progression of many growth factors. Further support of the stimulatory effect of 660 nm came from a study by Ghali et al. [31]. They showed that of the endothelial

cells exposed to 660 nm or 820 nm wavelengths from 15 mW GaAlAs laser diodes, only the 660 nm-irradiated cells were significantly higher than their controls, suggesting 660 nm LLL induced the release of mitosis-promoting cytokines and growth factors.

### Possible Mechanisms and Factors Affecting Postirradiation Cell Counts

The response of LLL on cells may be dose-dependent [14] as demonstrated on human fibroblasts [32], as well as wavelength-dependent [33,34]. Thus a 1.5–2-fold enhancement in the percentage of mitotic NIH/3T3 fibroblasts after cell exposure to 10 mW HeNe LLL within 1–10 J/cm$^2$ range was observed, whereas a similar effect was obtained using a 7 mW 660 nm light-emitting diode (LED) and a 7 mW 940 nm LED [32]. It was also confirmed that the HeNe LLL effect on the fibroblast proliferation was dependent on their enrichment by exogenous hematoporphyrin derivatives, whereas that of 940 nm was not [32]. This finding supports the hypothesis that visible red light is absorbed by the endogenous porphyrins of the mitochondria. Likewise, using in vitro rat Schwann cells, a wavelength-dependent effect by LLL was shown [34]. Even with an appropriate wavelength that can be absorbed by the targeted tissues, employing the correct power density, exposure time, and energy density are important parameters that require further evaluation [4,14,15]. As shown by Arndt-Schultz Curve and described by Ohshiro [14], appropriate LLL parameters and wavelength are necessary before a stimulatory effect can be obtained, e.g., in this study. In human fibroblasts, the absorption bands of their photoacceptors, presumably the respiratory chain pigments and endogenous porphyrins [35] along with their absorption peaks, have been studied, particularly of the visible light spectrum. Probably visible light is absorbed by both as mitochondrial cytochromes and porphyrins have similar action spectra [33]. On absorption of suitable optical energy, the basic mechanism that triggers off the cascade of molecular activities within the cell includes enhancement of mitochondrial proton motive force, which eventually leads to increased ATP synthesis.

Different wavelengths and parameters affect different photoacceptors present in the respiratory chain, the result of which may be a stimulatory or inhibitory effect [33]. The studies that demonstrate inhibitory or no responses in tissues that have been exposed to LLL red light or other appropriate wavelengths [2,6,36] would suggest that either their incident photoenergy was inadequate, or had exceeded the stimulatory range for inducing stronger biological activities in the cells. Thus the excessive cumulative effects of daily or even three times/day exposure to LLL over several days may have reversed any initial beneficial effects [14] of red light irradiation, which may have occurred in some studies [2,35]. It is unlikely that optimally growing cell cultures such as human embryonal fibroblasts can be stimulated further by LLL irradiation [2,6]. In this study, since the HSF and NDF cell counts were enhanced on all the 5 postirradiation days indicated the cells responded to the LLL parameters employed.

As the 660 nm wavelength is at the far red part of the visible light spectrum, it may bioactivate the ATPase ion pumps in the cell membrane [27] as well as at the mitochondria. Infrared LLL interactions occur at the irradiated cell membranes by cell chromophores, e.g., endogenous porphyrins [7], which then considerably alters the transmembrane transport of various cations, notably $Ca^{++}$. LLL-mediated increased cell membrane permeability to $Ca^{++}$ may trigger the chain of events associated with release of cytokines including growth factors and shifts of ions [32,34,35]. It has been reported that increased calcium uptake rates in macrophages following LLL were wavelength, dose, and pulse frequency-dependent [38].

Small amounts of singlet oxygen were generated in fibroblasts following their exposure to HeNe LLL [32]. At the respiratory chain level, visible light-mediated, reactive oxygen species, including singlet oxygen, probably modulate the mitochondrial redox activity. Although endogenous porphyrins are excellent photo-sensitizers through their generation of highly reactive singlet oxygen, the latter can damage in vivo tissue following inappropriate wavelength irradiation and despite using low energy densities [27,33]. However, small amounts of singlet oxygen photoproduced by neutral porphyrins or cytochromes may assist with DNA synthesis, redistribution of $Ca^{++}$ within the cell [32,35], and promote the $Ca^{++}$ influx into the cytoplasm necessary for promoting cell division [7,37].

In mitosis, some proto-onco-genes code for certain growth factors and receptors, whereas others do so for specific cytoplasmic or nuclear proteins. It was suggested that growth factors released through LLL mediation [9] facilitate at least part of the specific path through which cer-

tain growth factors prepare the cells for enhanced DNA synthesis and mitosis. Also observed in this process was an increase in succinic dehydrogenase alongside cell proliferation following LLL [8]. Succinic dehydrogenase is a reliable indicator of the cell respiratory status as it affects hydrogen transfer from succinic acid to mitochondrial cytochromes.

### Appropriate LLL Wavelength for Hypertrophic Wounds

The possibility of influencing fibroplasia in dermal healing by LLLT should point to the search for a wavelength that has an inhibitory effect in the treatment of hypertrophic wounds. Unlike normal wound healing where numerous reparative activities work in concert, mainly controlled by growth factors [20,21,25,26], this orderly control may be lacking in hypertrophic wounds. Apart from the primary effect, the secondary effects of LLLT are believed to have systemic effects, such as enhancing the immune system, or affecting tissues that are not at the LLL-tissue interaction site [13,14,35]. As an excess of FGFs is associated with neoplasia [22] and high levels of transforming growth factor β (TFF-β) produce scarring [18,25], the stimulatory effects of LLLT may be undesirable for burns and other wounds susceptible to hypertrophic scarring. Such scars have a significant increase than normal skin in mRNAs for procollagen types I and III, type I procollagen protein, and in TGF-β1 mRNA [11]. FGF-2 has been shown to stimulate granulation tissue fibroblasts to synthesize collagen and may compound the problem as the collagenase level in postburn hypertrophic scar fibroblasts was found to be lower than that of normal fibroblasts [39]. Thus fibroblast phenotypes should also be considered when assessing the effect of LLL on this cell type in future studies.

## CONCLUSION

This study found that 660 nm LLL from a 17 mW laser diode, pulsed at 5 KHz, at energy densities of 2.4 J/cm$^2$ and 4 J/cm$^2$ increased HSF and NDF cell counts. It is suggested that stimulatory red light wavelengths would be inappropriate for treating burn wounds that are susceptible to hypertrophic scarring. LLLT of wavelengths that could produce an inhibitory effect on wound fibroblast proliferation may be more suitable for such wounds. As the NDF cell numbers were also enhanced, the result also suggests that the 660 nm wavelength at the specified energy densities may accelerate the healing of indolent ulcers through increasing fibroblast cell numbers. Finally, more studies are required to unveil the mechanisms and relevance of LLL-induced growth factors in dermal repair; the results obtained would be of considerable clinical significance.

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